Natural Hormones
in Northern Ireland

Armstrong Hewitt & Glenn Kennedy
VSD Belfast
UK-NRL : Hormones and others
Legal cases are under way

Information is confidential

All information shown here:

In confidence!!
### Hormones covered by NI testing schemes

<table>
<thead>
<tr>
<th>ADD</th>
<th>Methyl Boldenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Boldenone</td>
<td>Methyltestosterone</td>
</tr>
<tr>
<td>β-Boldenone</td>
<td>Norethandrolone</td>
</tr>
<tr>
<td>CLAD</td>
<td>D(-) Norgestrel</td>
</tr>
<tr>
<td>Dienestrol</td>
<td>α-Nortestosterone</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>β-Nortestosterone</td>
</tr>
<tr>
<td>α-Estradiol</td>
<td>Progesterone</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>Stanozolol</td>
</tr>
<tr>
<td>Ethisterone</td>
<td>Testosterone</td>
</tr>
<tr>
<td>Fluoxymesterone</td>
<td>α-Trenbolone</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>Zeranol</td>
</tr>
<tr>
<td>16β-Hydroxy stanozolol</td>
<td>α-Zearaleneol</td>
</tr>
<tr>
<td>Medroxyprogesterone</td>
<td>β-Zearaleneol</td>
</tr>
<tr>
<td>Methenolone</td>
<td></td>
</tr>
</tbody>
</table>
How does the test work: 1

**Urine (5 ml)**

Treated with an enzyme (from *Helix pomatia*) to release hormone from conjugates

**Clean-up**

**LC-MS-MS**

**Confirmation**
Urine (5 ml)

Clean-up

LC-MS-MS

Confirmation

How does the test work: 2

- Automated clean up
- ASPEC system
- Two sequential SPE steps
- Run overnight
How does the test work: 3

Urine (5 ml) → Clean-up → LC-MS-MS → Confirmation

- Screen for 22 hormones/metabolites
- 18 minutes per sample
- 2 HPLC columns
- Run overnight
Urine (5 ml)

Clean-up

LC-MS-MS

Confirmation

- 20% of HPLC effluent enters MS-MS
- Remainder → fraction collector
- Fractions used for confirmation
- LC-MS-MS ; GC High Resolution MS ; GC-MSMS
Time Sectoring LC-MSMS Functions

Time sectoring of functions helps minimise cycle time

22 compounds + 13 deuterated internal standards

If all transitions were being monitored simultaneously the total cycle time would be approx 9 seconds.

This would give at best 3 measurements across a 30 second wide peak.
A limit of detection of 0.5 ppb or less is desirable.

On the Quattro LC this can only be achieved for these compounds if dwell times of greater than 0.1 of a second are used for each transition product.
Quattro LC MS method contains maximum number of transitions possible

9 scans across peak

Medroxy progesterone
To make this method confirmatory would necessitate increasing the number of transitions measured to 53 i.e. two for each hormone + one for each internal standard.

This would result in a maximum of 4 - 5 scans across each peak.

Serious risk of missing peak top

Would negatively impact on ion ratio repeatability
OVERLOAD!!!!
Quattro Premier
**Short dwell times possible**

<table>
<thead>
<tr>
<th>Dwell</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 Sec</td>
<td>763500</td>
</tr>
<tr>
<td>0.10 Sec</td>
<td>868438</td>
</tr>
<tr>
<td>0.05 Sec</td>
<td>878869</td>
</tr>
<tr>
<td>0.025 Sec</td>
<td>878661</td>
</tr>
<tr>
<td>0.010 Sec</td>
<td>871395</td>
</tr>
</tbody>
</table>

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5 ppb Solvent Std Dwell 0.25:
- Steroids74: 345.3 > 95.8
  - F1: 434.1
  - Area: 4.93e6

5 ppb Solvent Std Dwell 0.10:
- Steroids75: 345.3 > 96.8
  - F1: 5.84e6
  - Area: 6.19e6

5 ppb Solvent Std Dwell 0.05:
- Steroids76: 345.3 > 96.8
  - F1: 5.84e6
  - Area: 6.19e6

5 ppb Solvent Std Dwell 0.025:
- Steroids77: 345.3 > 96.8
  - F1: 5.84e6
  - Area: 6.19e6

5 ppb Solvent Std Dwell 0.010:
- Steroids78: 345.3 > 96.8
  - F1: 5.84e6
  - Area: 6.19e6
Improves No. of scans across the peak

<table>
<thead>
<tr>
<th>Dwell</th>
<th>Scans</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 Sec</td>
<td>24</td>
</tr>
<tr>
<td>0.10 Sec</td>
<td>49</td>
</tr>
<tr>
<td>0.05 Sec</td>
<td>86</td>
</tr>
<tr>
<td>0.025 Sec</td>
<td>130</td>
</tr>
<tr>
<td>0.010 Sec</td>
<td>220</td>
</tr>
</tbody>
</table>

5 ppb Solvent Std Dwell 0.25
Steroids 74
F1: 345.3 > 96.8
Area: 4.93e6

5 ppb Solvent Std Dwell 0.10
Steroids 75
F1: 345.3 > 96.8
Area: 6.19e6

5 ppb Solvent Std Dwell 0.05
Steroids 76
F1: 345.3 > 96.8
Area: 5.69e6

5 ppb Solvent Std Dwell 0.025
Steroids 77
F1: 345.3 > 96.8
Area: 5.84e6

5 ppb Solvent Std Dwell 0.010
Steroids 78
F1: 345.3 > 96.8
Area: 6.04e6
Premier - 0.01 sec dwell times

Dwell times of 0.01 seconds for all transition products

57 transition products monitored with no attempt to time sector functions (equating to 2 transitions for each steroid + 13 internal standards)
57 transition products monitored

Dwell time 0.01 seconds

48 scans across each peak

15 scans per peak sufficient
Quattro Premier- HPLC Method

27 Analytes + 13 Deuterated ISTD
77 Transition ions monitored
150 X 2.1 mm HPLC Column
HPLC run time 16 minutes
9 minutes for re-equilibration

Total run time 25 minutes
**CC$\alpha$ and CC$\beta$ - selected compounds**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>CC$\alpha$</th>
<th>CC$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Boldenone</td>
<td>0.50</td>
<td>0.85</td>
</tr>
<tr>
<td>$\beta$-Boldenone</td>
<td>0.41</td>
<td>0.70</td>
</tr>
<tr>
<td>$\alpha$-Estradiol</td>
<td>N.D. *</td>
<td>N.D. *</td>
</tr>
<tr>
<td>$\alpha$-Nortestosterone</td>
<td>0.37</td>
<td>0.62</td>
</tr>
<tr>
<td>$\beta$-Nortestosterone</td>
<td>0.77</td>
<td>1.31</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.49</td>
<td>0.84</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.52</td>
<td>0.88</td>
</tr>
</tbody>
</table>

* Poor sensitivity on Quattro Premier*
Define a normal range in a normal population

Urine samples (94) collected
Castrated male cattle
Government farms
Trustworthy farmers (!!)
Tested by LC-MS/MS

What is a non-compliant result?

Identified and greater than \( \text{CC}_\alpha \)
Natural hormones??

Define a normal range in a normal population

Urine samples (94) collected
Castrated male cattle
Government farms
Trustworthy farmers (!!)
Tested by LC-MS/MS
What is an abnormal level?

The easy ones ...
In our animal population (mix of breeds)
Under our dietary system (grass and grass silage based)
Animals 24-30 months of age

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ - Boldenone</td>
<td>None detected</td>
</tr>
<tr>
<td>$\beta$ - Boldenone</td>
<td>None detected</td>
</tr>
<tr>
<td>$\alpha$ - Nortestosterone</td>
<td>None detected</td>
</tr>
<tr>
<td>$\beta$ - Nortestosterone</td>
<td>None detected</td>
</tr>
</tbody>
</table>
However...

We have found boldenone in our control schemes
Cattle and sheep
EU guidelines for interpretation - Veal calves

Conjugated $\beta$-boldenone
Abuse
Conjugated $\alpha$-boldenone $> 2$ ppb,
Suspicious
Unconjugated $\alpha$- or $\beta$-boldenone
Faecal contamination
(on-farm samples ?)

Our findings - “suspicious”
Nothing at on-farm follow-up
Progesterone detectable in all 94 samples

Highest concentration 3.49 µg/ L

Normal

Natural log

Positive skew
Significant P < 0.01

Negative skew
Not significant (P > 0.05)
## Progesterone

**Tentative Upper Limit of normality**

Steers, Northern Ireland, grass-fed, etc, etc

<table>
<thead>
<tr>
<th>Statistical Certainty</th>
<th>Upper Limit (µg/ L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99 %</td>
<td>4.2</td>
</tr>
<tr>
<td>99.9 %</td>
<td>8.4</td>
</tr>
<tr>
<td>99.99 %</td>
<td>14.7</td>
</tr>
</tbody>
</table>
Testosterone

Detectable in half of samples

Highest concentration 2.41 µg/L

*Very* tentative Upper Limit of normality

Steers, Northern Ireland, grass-fed, etc, etc

Strong positive skew to data (lower LoD would help)

<table>
<thead>
<tr>
<th>Statistical Certainty</th>
<th>Upper Limit (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99 %</td>
<td>1.8 *</td>
</tr>
<tr>
<td>99.9 %</td>
<td>5.0</td>
</tr>
<tr>
<td>99.99 %</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* 1/94 samples > 1.8 µg/L
$17\alpha$-estradiol

93/94 Negative

One positive 1.2 µg/L

Repeat: Micromass XE (negative mode)

Other confidential data

Steers, n=106

Suggested upper limit of normal (99 %) = 3.8 µg/L
Current problems *(not for publication)*

Meat Inspection Scheme *(suspects)*

Three steers samples

One steer condemned

$17\alpha$-19-nortestosterone (1.2 µg/ L)

DARD/ Police raid

95 animals seized and destroyed
## Laboratory findings

<table>
<thead>
<tr>
<th>Description</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabolic preparations</td>
<td>none found</td>
</tr>
<tr>
<td>Injection sites</td>
<td>none found</td>
</tr>
<tr>
<td>Syringes</td>
<td>nothing illegal</td>
</tr>
<tr>
<td>Urine from steers</td>
<td>no synthetics progesterone</td>
</tr>
<tr>
<td>Hair from steers</td>
<td>no corticosteroids no hormone results</td>
</tr>
</tbody>
</table>
Progesterone

No -OH groups
No esters!
Group of animals

Progesterone (µg/kg)

94 Normal Steers
Group of animals

Progesterone (µg/kg)

94 Normal Steers

Upper limit Normal Range 99%
In Northern Ireland

Hormone preparations still in use
No preparations available
Looking for a “needle in a hay stack”
Natural hormones a major constituent
Need to establish normal ranges validity of esters in hair IRMS
Willing to participate in any initiatives